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Conversion of Acetaldehyde-Protein Adduct Epitopes From a Nonreduced to a Reduced Phenotype by Antigen Processing Cells

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Many investigators have suggested that an immune reaction to acetaldehyde-protein adducts may be involved in the development and/or progression of alcohol liver disease. The most often reported acetaldehyde adduct is the reduced adduct prepared in vitro in the presence of strong reducing agents. However, the production of this adduct in vivo has been difficult to prove. Nevertheless, the detection of serum antibodies to this reduced adduct following alcohol exposure in animals and humans has been used to support the formation of this adduct in vivo. We have recently observed that when acetaldehyde-protein adducts prepared under nonreducing conditions are used to immunize animals, antibody to the reduced protein adduct is detected. Therefore, it was the purpose of this study to demonstrate that nonreduced (NR) adduct epitopes can be modified by intact cells to express reduced (R) adduct epitopes. This was accomplished using the monoclonal antibody RT1.1 that has been previously characterized by this laboratory and has been shown to recognize only R and not NR acetaldehyde adducts. In these studies, Balb/c mice were injected intraperitoneally (500 μg/animal) with either keyhole limpet hemocyanin (KLH)-NR or KLH-R adducted proteins. Immunization with KLH-NR produced significant amounts of antibodies that recognized both NR and R epitopes. In contrast, immunization with KLH-R produced antibodies to only R and not NR epitopes. Isolated peritoneal macrophages from nonimmunized mice were incubated in vitro with either KLH-NR, KLH-R, or unmodified KLH proteins, and the cell surface expression of the reduced epitope (RT1.1) and the activated macrophage marker (MAC-3) determined by double immunofluorescent staining. Activated macrophages incubated with KLH-NR expressed the R adduct on 11.5% of the cells, compared with 3.8% following incubation with unmodified KLH, and 19.4% following incubation with KLH-R. These data suggest that the NR adduct and/or the carrier protein are modified by peritoneal macrophages in vivo and present an epitope that is detected as a reduced adduct (RT1.1 positive). These observations may explain the presence of circulating antibodies to the reduced adduct that has been reported in human and animal studies.

Key Words: Alcohol, Acetaldehyde, Antigen Processing Cells, Monoclonal Antibodies, Peritoneal Macrophages.

ALCOHOLIC LIVER disease (ALD) remains a major worldwide health problem for which no specific pathophysiology has been defined. The possibility that covalent binding of acetaldehyde (AA) to proteins may contribute to liver injury was originally postulated by Sorrell and Tuma (1985). Evidence has accumulated that suggests AA binding to proteins in vivo alters protein and cellular function (Smith et al., 1989). Although these AA adducts may alter cellular functions by direct toxicity, it has become clear that antibodies to these adducts can be produced and may result in immune-mediated tissue damage. Antibodies to some of these adducts have been detected in the serum of patients who chronically consume alcohol (Niemala et al., 1987; Worrall et al., 1990; Viitala et al., 1997). Additionally, it has been reported that AA adducts recognized by these antibodies have been detected in the livers of patients with ALD (Moretta et al., 1993; Niemala et al., 1991).

While increasing evidence suggests that immune recognition of, and response to, AA protein adducts occurs in vivo, it is unclear which adducts are biologically important. That is, at least two types of AA adducts have been reported: (1) the nonreduced (NR) adduct that is a stable adduct prepared in the absence of sodium cyanoborohydride ($NaCNBH_3$) (Tuma et al., 1991b); and (2) an unsta-

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ble adduct that is stabilized in the presence of the strong reducing agent $NaCNBH_3$ (Tuma et al., 1991b). The current literature on immune responses to AA protein adducts contains experimental approaches that result in the production of antibodies to a wide variety of unique AA protein adducts (Niemala et al., 1991; Lin et al., 1988; Worrall et al., 1991b). Most studies have used high concentrations of AA (>100 mM) in the presence of reducing agents to form an AA protein adduct that was then utilized as the immunogen. A recent report from our laboratory using a monoclonal antibody (RT1.1) to the reduced epitope, *N*-ethyl-lysine (NEL), showed that these adducts are not found when AA protein adducts are prepared under physiological or nonreducing conditions (Thiele et al., 1994; Klassen et al., 1994). Additionally, these adducts could not be detected in the livers of rats chronically fed alcohol using RT1.1 as a probe. However, it was observed that, when animals were immunized with AA protein adducts prepared under nonreducing conditions, antibodies that recognized both the NR as well as the R protein adducts were detected (Klassen et al., 1992). This occurred despite the observation that R (NEL) epitopes cannot be detected on NR adducts. Therefore, it was the purpose of these studies to demonstrate that either specific and/or unique modifications of the chemical structure of AA protein adducts occurs during antigen processing in vivo, or the cleavage of AA-modified proteins by antigen presenting cells (APCs) results in the presentation of an epitope that can be recognized by an antibody to the R adduct, NEL. These data could explain the presence of serum antibodies to the reduced adduct (NEL) in the absence of the production of this adduct in tissues after alcohol ingestion.

METHODS

Antigen Preparation

Bovine serum albumin (BSA) (crystallized, lyophilized, and fatty acid-free) and keyhole limpet hemocyanin (KLH) was purchased from Calbiochem (San Diego, CA). Tubulin was prepared by the method of Shelanski et al. (1973), and rabbit actin was prepared as described by Carsten and Mommaerts (1963). Rabbit plasma protein (RbPP) or rat plasma protein (RtPP), was prepared from whole serum as previously described (Worrall et al., 1990; Klassen et al., 1993). Briefly, serum proteins were precipitated by adding 70% ammonium sulfate overnight at 4°C. The insoluble material was pelleted at 10,000 × *g*, supernatant discarded, and the pellet rehydrated with double distilled water.

Rabbit liver cytosol (RbLC) and rat liver cytosol (RtLC) were prepared by the method of Touster et al (1985). Briefly, liver cytosols were prepared by homogenizing the livers in a Dounce homegenizer with 2 to 3 ml of phosphate-buffered saline (PBS). The homogenates were centrifuged in a Beckman JA-21 ultracentrifuge (Beckman Instruments, Palo Alto, CA) at 10,000 × *g* for 20 min to remove membranes. The supernatants were further centrifuged at 100,000 × *g* for 1 hr in a Beckman ultracentrifuge to obtain predominantly cytosolic proteins.

All proteins were either brought up in or dialyzed overnight against 0.1 M phosphate buffer. Protein concentrations were determined by Groves et al. (1968). Proteins were modified with AA under reducing conditions by adding either 5 mM AA and 30 mM $NaCNBH_3$ (5/30) or 240 mM AA for 1 hr and 100 mM $NaCNBH_3$ (240/100) for 30 min to 1 ml of protein at 1.2 mg/ml. Proteins were modified under nonreducing conditions by the addition of 100 mM AA to 1 ml of protein at 1.2 mg/ml. In each case, the solutions were incubated at 37°C overnight, dialyzed against three changes of PBS for 8 hr, and used for coating ELISA plates. Native unmodified proteins were used as controls.

Although these levels are higher than those found under physiological conditions in the liver after alcohol ingestion, they are still much lower than other investigators have reported (Worrall et al., 1991b; Stevens et al., 1981; Israel et al., 1986; Hoerner et al., 1988). Additionally, under nonreducing conditions, we have shown that 5 mM AA incubated for 5 days at 37°C produces an adduct similar to that produced by 100 mM AA overnight at 37°C (Klassen et al., 1994). Therefore, in an attempt to standardize these assays, we incubated each adduct overnight. Also, to ensure the proteins were not aggregated, all proteins were centrifuged at 100,000 × *g* for 30 min and protein concentrations reassessed using the method of Groves et al. (1968).

Antibody Activity to Modified Proteins

Proteins to be tested were diluted to 20 μg/ml in bicarbonate buffer (pH 9.6) and coated on ELISA plates overnight at 37°C with 100 μl of the test protein. To the washed plates, 200 μl of either serum or monoclonal supernatant were added to row B and diluted 2-fold down the plates. The plates were incubated at 37°C for 45 min, washed three times with PBS-Tween 20 and 100 μl of alkaline phosphatase-labeled rabbit anti-mouse IgG, IgM, and IgA (H&L), or 100 μl of alkaline phosphatase-labeled goat anti-rabbit IgG (H&L) was added. Both antibodies were purchased from Zymed Laboratories (South San Francisco, CA) and used at a 1:1,000 dilution. After a 45-min incubation at 37°C, the plates were washed three times with PBS-Tween 20 and 100 μl of *p*-nitrophenyl phosphate in diethanolamine buffer was added. The plates were monitored at 405 nm on a DynaTech MR7000 MicroELISA Reader after 30 min of incubation. In some cases the optical density of representative assays were reported. However, whenever possible, the geometric mean titer (GMT) of antibody dilutions was reported that represented the average of five separate determinations.

The reactivity of RT1.1 was assessed by ELISA using: KLH, BSA, bovine brain tubulin, rabbit muscle actin, RtLC, RtPP, RbLC, and RbPP. These proteins were unmodified (native), or were modified under reducing or nonreducing conditions. The plates were coated at 20 μg/ml and RT1.1 (positive control) or an isotype (negative control) control was added for 45 min at 37°C. The plates were washed 3 times and then alkaline phosphatase-labeled rabbit anti-mouse IgG, IgM, and IgA (H&L) diluted at 1:500 were added and incubated for 45 min at 37°C. The plates were washed three times, substrate added, and read as above.

Immunizations

Balb/c mice were obtained from the National Cancer Institute via an interagency agreement with the Veteran's Administration and maintained on water and laboratory chow ad libitum. All animals received humane care in compliance with the institution's guidelines and the National Research Council's criteria for humane care as outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences (1985) and published by the National Institutes of Health. The mice were quarantined immediately on arrival and monitored on a monthly basis to ensure specific pathogen-free status. Five mice were injected intraperitoneally three times over a period of 6 weeks with 100 μg of unmodified (native) or modified (NR or R), KLH in Freund's complete (week 1) or Freund's incomplete (weeks 3 and 5) adjuvant. Animals were bled 2 weeks after the last immunization and tested for antibody titers to proteins modified under native, NR, or R conditions.

Isolation of Peritoneal Macrophages

Balb/c mice were injected with 1 ml of sterile thioglycollate broth (Difco Laboratories, Detroit, MI), and 5 days later the animals were killed. The peritoneal exudate cells were obtained by injection and extraction of 9 ml of ice-cold Hanks' balanced salt solution (HBSS) containin

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10 units of heparin/ml. Cells were centrifuged at 250 × *g* for 5 min at 4°C, resuspended, and counted with a hemocytometer. Cells were adjusted to 1×10^6/ml and plated into 24 well plates at 1 ml/well. The plates were incubated for 60 min at 37°C in HBSS (serum-free); washed; and 1 mg of KLH, KLH-NR, or KLH-R was added to appropriate wells and incubated at 37°C. At the end of 4 hr, cells were harvested by scraping, adjusted to 1×10^6 cells/ml, and 1 ml was added to appropriately labeled tubes for FACScan staining.

Modification of AA Adduct Epitopes by Macrophages

The peritoneal exudate macrophages were pelleted, and stained for the presence of the reduced adduct using RT1.1 (NEL specific) and the macrophage marker MAC-3. Briefly, 1×10^6 cells were incubated with 2 μg of rat anti-FcγIII receptor antibody (Pharmingen, San Diego, CA) to inhibit nonspecific binding to Fc receptors on macrophages. Cells were then incubated with either RT1.1, phycoerythrin (PE)-labeled MAC-3 or the appropriate isotype controls. After incubation with the first antibody for 45 min on ice, the cells were washed three times and incubated with the fluorescein isothiocyanate-labeled rat anti-mouse IgG2b second antibody purchased from Zymed Laboratories, Inc. After the final wash, the cells were brought up in 1.0 ml of FACS fixative (2% paraformaldehyde in Dulbecco's PBS). Cells were analyzed using a FACScan (Becton Dickinson, San Jose, CA) and LYSYS software.

Specificity of Antibodies for FACScan Staining

The presence of the R adduct (NEL) was detected by using the RT1.1 mouse monoclonal antibody (IgG2b isotype) prepared in this laboratory. The production and specification of RT1.1 has been previously described (Thiele et al., 1994; Klassen et al., 1994). Although RT1.1 resulted from an immunization with NR protein, it recognizes only proteins modified under reduced conditions, but not native proteins or those modified under nonreducing conditions. A mouse IgG2b myeloma was purchased from Sigma Chemical Co. (St. Louis, MO) to serve as the negative control for RT1.1.

The PE-labeled MAC-3 antibody, which is thought to recognize CD107b found on tissue macrophages and thioglycollate-elicited peritoneal macrophages (Johnson et al., 1983; Adams and Hamilton, 1984; Springer, 1981; Ho and Springer, 1983; Flotte et al., 1983; Chen et al., 1985), was purchased from Pharmingen. To serve as a negative control for MAC-3, a PE-labeled rat IgG1 myeloma was purchased from Pharmingen.

ELISA to Assess the Affects of Freund's

To determine whether the antibody activity observed using the FACScan was due to the reduced adduct and not to an artifact of Freund's complete adjuvant, an ELISA was run by coating plates with the AA-modified antigens and antigens plus Freund's complete adjuvant. The plates were probed with either rabbit anti-KLH (1:1,000; ICN Biologicals, Naperville, IL) followed by alkaline phosphatase-labeled goat anti-rabbit IgG (H&L) diluted 1:500 (Zymed Laboratories). Duplicate plates were probed with RT1.1 (undiluted), and alkaline phosphatase-labeled rabbit anti-mouse IgG, IgM, and IgA (H&L) diluted 1:500 (Zymed Laboratories). Substrate was added and optical densities determined at 405 nm using a DynaTech MR7000 MicroELISA Reader after 30 min of incubation.

RESULTS

Antibody Response to Proteins Modified with AA Under Nonreducing Conditions

In mice immunized with proteins modified under nonreducing conditions (Fig. 1A), serum antibodies to both the NR adduct (GMT of ~2) and the R adduct (GMT of ~4) were observed. This was despite the fact that the R adduct

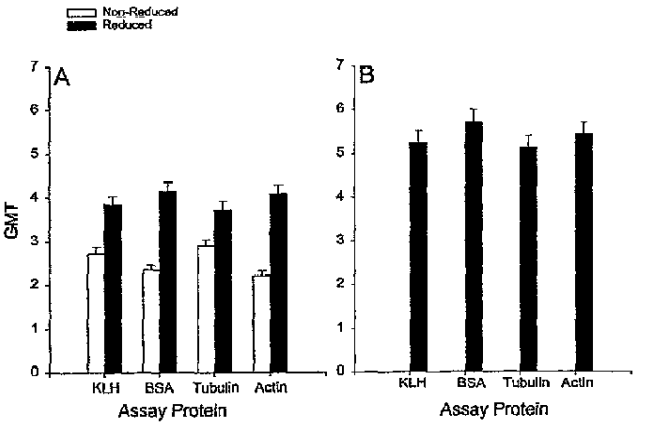

Fig. 1. Detection of serum antibodies to NR and R adducts after immunization of mice with KLH, BSA, tubulin, or actin modified under: (A) NR or (B) R conditions. Antisera were tested on the immunizing protein modified with nothing or AA under nonreducing (NR) conditions, or AA under reducing (R) conditions. Activity to the native protein was subtracted from the activity to the NR (□) and R (■) adducts on the corresponding protein to assess the amount of antibody that was specific for the adduct and not the protein. The GMT of five animals from each group are reported ± SEM.

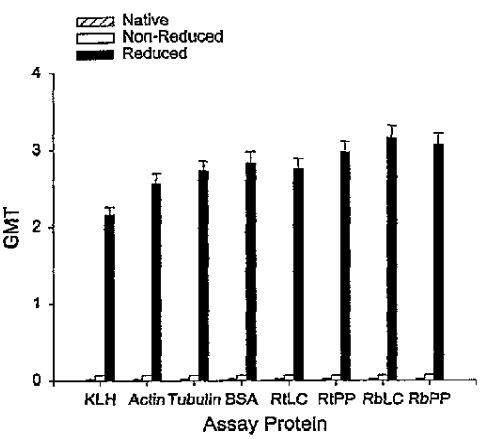

Fig. 2. The reactivity of RT1.1 to proteins modified with AA under: (1) Native (▨), (2) NR (□), and (3) R (■) conditions. The proteins assayed included: KLH, actin, tubulin, BSA, RtLC, RtPP, RbLC, and RbPP. The GMT of five animals from each group are reported ± SEM.

was never detected on proteins modified under nonreducing conditions with either a polyclonal antibody affinity purified to NEL (data not shown) or the monoclonal antibody RT1.1 (Fig. 2). In contrast, immunization with proteins modified with AA under reducing conditions demonstrated antibody activity to only the reduced adduct at GMTs of ~5.0 (Fig. 1B). Finally, little or no activity to the NR adduct or R adduct was detected when animals were immunized with unmodified proteins (data not shown).

Monoclonal Antibody Response to Protein Adducts Produced by Immunization with NR Protein Adducts

Immunization of a Balb/c mouse with NR protein adducts, and fusion with NS-1 myeloma cells resulted in one

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Table 1. Antibody Activities to AA-Modified Proteins in the Presence/Absence of Freund's Complete Adjuvant (FCA) by ELISA

Antibody	KLH		KLH-NR[a]		KLH-R[b]	
	No FCA[c]	With FCA[d]	No FCA	With FCA	No FCA	With FCA
Rb × KLH[e]	3.26[f]	3.21	3.87	3.89	3.92	3.87
RT1.1[g]	<0.1	<0.1	<0.1	<0.1	2.69	2.17

[a] KLH-NR was prepared using 100 mM AA at 37°C for 16 hr.
[b] KLH-R was prepared using 5 mM AA and 30 mM $NaCNBH_3$ at 37°C for 16 hr.
[c] Original antigens were diluted 1:20 in sodium bicarbonate buffer for coating.
[d] Antigens were diluted 1:20 in sodium bicarbonate buffer containing 25% Freund's Complete adjuvant.
[e] Rabbit anti-KLH antiserum.
[f] Optical density at 405 nm. This is the average of triplicate samples and is representative of three different assays.
[g] RT1.1 is a monoclonal antibody that only recognizes AA-modified protein adducts prepared under reducing conditions.

clone that produced antibody that recognized most proteins modified with R adducts (Thiele et al., 1994; Klassen et al., 1994). The activity of the RT1.1 monoclonal antibody was assessed by ELISA to unmodified (native), NR, or R proteins: KLH, rabbit muscle actin, BSA, RtLC, RtPP, RbLC, and RbPP. In every instance, RT1.1 was found to detect only those proteins prepared under reducing conditions (Fig. 2). Recently, we have demonstrated that RT1.1 is specific for NEL residues that have been suggested to be one of the major products formed under reducing conditions (Thiele et al., 1994). It is important to note that, although RT1.1 is the product of immunizing with NR adducts, it does not bind to AA protein adducts prepared in vitro under nonreducing conditions.

Effect of Freund's Complete Adjuvant on Proteins

It was possible that Freund's complete adjuvant may have modified the NR proteins in vivo such that the RT1.1 recognized it as a R adduct. To assess this, we coated ELISA plates with either the antigen or the antigen mixed with 25% Freund's complete adjuvant. The activity was assessed using both RT1.1 and a rabbit anti-KLH antibody (Table 1). Using the rabbit anti-KLH antibody, it was possible to show that the adjuvant had no effect on the coating and antigenicity of the KLH. Likewise, the binding of RT1.1 to KLH-R was only moderately decreased. Of importance, exposure of the KLH-NR to Freund's adjuvant did not alter the epitopes, nor allow for the nonspecific binding of RT1.1.

FACscan Analysis of Peritoneal Cells

Peritoneal macrophages from each group of mice were analyzed by dual staining for MAC-3 and RT1.1 activity. Using the LYSYS software package, cells were analyzed for these two markers using FL-1 (RT1.1) versus FL-2 (MAC-3) dot plots. In these studies, immunization with PBS or unmodified KLH in Freund's complete adjuvant resulted in the detection of 16.1% and 13.2% (upper left quadrant) MAC-3 positive cells (Fig. 3A and 3B, respectively). However, little or no RT1.1 staining was observed as seen in the lower right quadrant of Fig. 3A and 3B. In contrast, exposure to KLH-R resulted in 19.4% of the cells staining with MAC-3, and this same population of cells (upper right quadrant) stained with RT1.1 (Fig. 3C). It appears an additional 15.7% of these cells also stained with RT1.1 only (lower right quadrant). In contrast, KLH-NR exposed cells did not express RT1.1 only (lower right quadrant). But, 11.5% of the KLH-NR cells stained with both MAC-3 and RT1.1 (Fig. 3D). Thus, exposure of the NR epitope appears to result in the expression of R epitopes on MAC-3 positive cells, suggesting that antigen processing and presentation must occur in order for the epitope to be expressed. In each case, the appropriate isotype control was run and did not result in any binding that was above that of PBS exposed cells. Peritoneal cells from mice injected with only Freund's complete adjuvant or KLH and Freund's complete adjuvant demonstrated no increase in staining with RT1.1 (data not shown).

DISCUSSION

ALD is a major medical concern worldwide, and many biochemical and immunochemical mechanisms of tissue damage have been proposed (Smith et al., 1989; Moretta et al., 1993; Tuma et al., 1987a, 1987b, 1991a, 1991c). However, the pathogenesis of this disease remains unknown. Recent work has shown that AA, the major metabolic product of alcohol oxidation, can bind to proteins and make them antigenic (Tuma et al., 1991b; Lin et al., 1988; Israel et al., 1988; Nicholls et al., 1992). There are two AA adducts that have been routinely reported in the literature. The first adduct is the stable product produced when AA binds a protein under physiological conditions, termed NR adducts (Tuma et al., 1991b). The second type occurs when unstable Schiff bases are stabilized by the addition of strong reducing agents (such as $NaCNBH_3$). Many, if not most of these R adducts are formed on epsilon-amino lysine groups and form NEL (Tuma et al., 1991b). It is important to note that other R adducts may be formed in vivo, but are not the focus of this study (Thiele et al., 1993, 1998).

Many investigators have demonstrated that R adducts can be immunogenic (Tuma et al., 1991b; Lin et al., 1988; Israel et al., 1988; Nicholls et al., 1992) and that patients with ALD, as well as experimental animals chronically fed alcohol, have serum antibodies to R adducts (Niemala et al., 1987; Worrall et al., 1990, 1991a, 1993; Viitala et al., 1997). However, these studies were all performed using in

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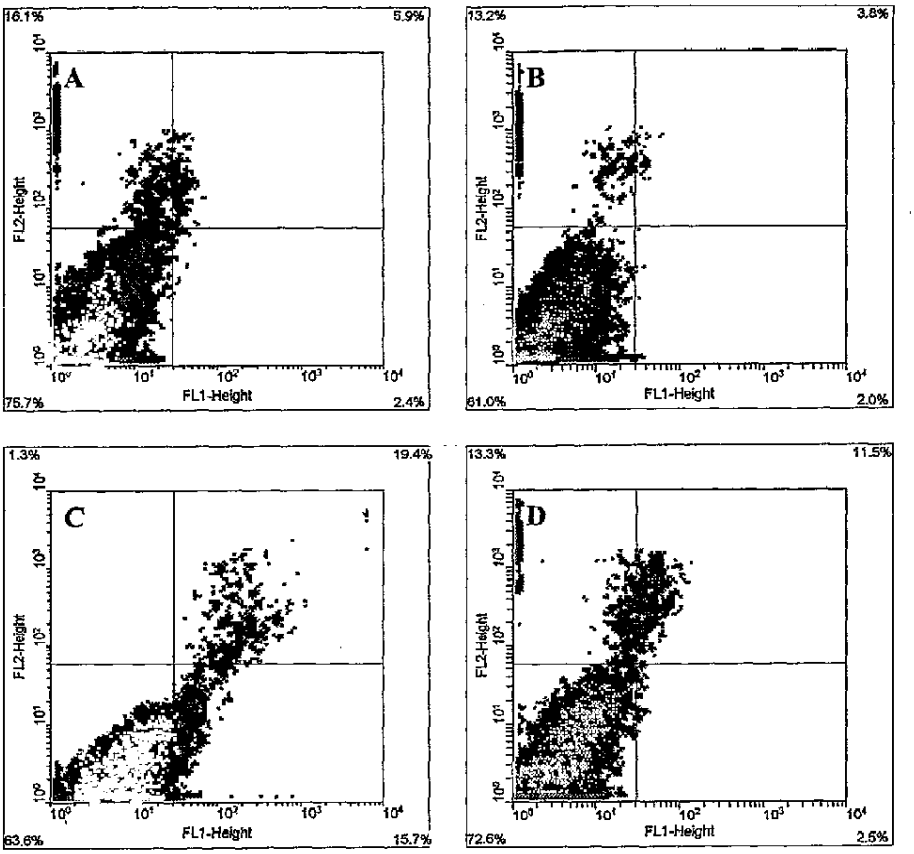

Fig. 3. FACScan analysis of peritoneal cells from Balb/c mice injected with thioglycollate broth and incubated with 1 mg of: (A) PBS, (B) KLH, (C) KLH-R, and (D) KLH-NR. Cells were stained for the presence of cell surface R adducts using RT1.1 followed by an FITC-labeled anti-mouse IgG2b second antibody, and PE-labeled MAC-3. Those cells that were only RT1.1 positive were detected by FL-1 and are found in the lower right quadrant. Cells that were MAC-3 positive (FL-2) only are found in the upper left quadrant. Cells that are positive for both RT1.1 and MAC-3 are found in the upper right quadrant, with the double negative cells in the lower left quadrant. Results are expressed as the % of 10,000 total cells positive for each antibody. The isotype controls did not show levels above those observed for PBS-exposed cells (A).

vitro modified proteins, and the mechanism whereby the R adduct can be produced in vivo is initially problematic. Recently, it has been suggested that immunization with the NR adduct unexpectedly results in the production of antibody to R (NEL) adducts (Klassen et al., 1992). Therefore, it was the purpose of this study to investigate the in vivo production of antibody to the R adduct NEL, after immunization with proteins modified under NR conditions.

In these studies antibodies recognizing R adduct epitopes were produced after immunization with proteins modified under nonreducing conditions. This was consistent with our original study wherein a unique monoclonal antibody (RT1.1) that recognizes only R (NEL) adducts that were cloned from splenic cells after immunization with NR adducts. Further studies have demonstrated that RT1.1 does not recognize the original immunizing protein adduct epitope. Thus, because the monoclonal antibody RT1.1—which has been shown to react with only NEL—was unable to bind to the immunizing proteins modified under NR conditions (Thiele et al., 1994; Klassen et al., 1994), it is unlikely that these proteins would result in the production of anti-R antibodies without some modification of the immunogen in vivo. These data strongly suggested that some in vivo mechanism exists to convert NR adduct epitopes to R adduct epitopes and may be due to the cellular processing of proteins adducted with NR adducts, resulting in the presentation of R adduct epitopes on the macrophage cell surface.

Initial studies were performed to determine if exposure to Freund's complete adjuvant could alter the expression of proteins modified under NR conditions in vitro, creating an epitope that mimics R adducts on the soluble protein. By mixing the adjuvant with the NR protein adduct, and assaying by ELISA, it was not possible to show R adduct formation in vitro. Therefore, some other mechanism must be converting the immunizing epitope from NR to R adducts in vivo and allowing the expression of this new epitope on the surface of APCs.

Experiments were performed using peritoneal macrophages as APCs to determine if the R adducts were formed after uptake and processing of NR protein adducts. In these studies it was observed that RT1.1, which only reacts with NEL, was able to detect R adducts on the surface of APCs from the peritoneum after incubation with NR adducts. Double staining experiments showed that cells that were positive for RT1.1 were also positive for activated macrophages (MAC-3 marker). In the case of the R adduct, the number of RT1.1 and MAC-3 positive cells were

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slightly higher than with the addition of NR adduct. Additionally, there was a percentage of cells that were positive for only RT1.1 and not MAC-3. Thus, it is possible that cells other than those that are MAC-3 positive either process and present the R (NEL) epitope, or the MAC-3 epitope has not been upregulated to this point. However, it is interesting to note that the presence of RT1.1 positive staining is only found in conjunction with MAC-3 when KLH-NR was introduced into the system. Regardless, these data strongly suggest that after the binding, processing, and presentation of NR adducts by APCs, there is the expression of the R adduct on the surface of the APCs that was not detected on the original immunizing protein-NR complex. Additionally, for an antibody to be produced that recognized this R epitope, some of the in vivo protein that now has the R epitope must bind to B cells that have surface immunoglobulin receptors for the R adduct.

The exact mechanism whereby proteins modified under NR conditions are ethylated once they are taken up by the peritoneal macrophages is unknown. Tuma et al. (1991b) have demonstrated that AA bound to a protein under the nonreduced conditions outlined in this study are extremely stable. Additionally, the RT1.1 antibody has never been able to detect the R adduct on these proteins prepared under NR conditions (Thiele et al., 1994; Klassen et al., 1994). However, all of these studies have been performed in vitro, and it is unknown what happens to these proteins after in vivo processing and presentation by APCs. Multiple possibilities exist to explain the conversion for NR to R epitopes during the internalization and processing of the modified protein: (1) some of the AA that is bound to the protein under NR conditions is released and reassociates with the protein under conditions that cause ethylation to occur; (2) the R epitope is present on the protein during modification under NR conditions, but the RT1.1 antibody is unable to detect it due to conformational constraints; and (3) the R epitope is the result of stabilization of Schiff bases inside the cell as has been previously reported (Lin et al., 1995). Regardless, these studies clearly demonstrate that immunization with NR adducts results in the expression of NEL epitopes on APCs, and that antibodies are produced to these in vivo altered adducts.

We speculate that the circulating antibodies to R epitopes that are seen after ethanol exposure may represent an epiphenomenon produced by this in vivo conversion of NR to R adduct. Thus, the in vivo presence of immune responses to the R adduct after alcohol consumption does not prove that the R adduct is formed directly as a consequence of alcohol metabolism. Further studies to determine the cellular mechanism used to convert NR to R adducts would help explain the presence and relevance of antibodies to these adducts in patients that consume alcohol. It is possible that the role of the R adduct in the pathophysiology of ALD is very limited, based on the observation that one of the main epitopes of the R adduct (NEL) is not seen in liver tissue. Additionally, the presence of anti-R adducts in vivo can be explained by the conversion of NR adducts. We suggest that the conversion of a highly relevant antigen (NR adduct) to an irrelevant one (R adduct) would be one mechanism by which tissue damage may be attenuated.

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